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Original Communication

Metabolic interaction between ethanol, high-dose alprazolam and its two main metabolites using human liver microsomes *in vitro*

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Abstract

Alprazolam is widely used as a short-acting antidepressant and anxiolytic agent and its effect appears at very low doses while ethanol is used as a social drug worldwide. Sometimes, toxic interactions occur following combined administration of these two drugs. In this study we have investigated the interaction between ethanol and high-dose alprazolam using human liver microsomes *in vitro*.

The interaction effects between ethanol and alprazolam were examined by a mixed-function oxidation reaction using a human liver microsomal preparation. Alprazolam and its two main metabolites (α-hydroxyalprazolam: α-OH alprazolam, 4-hydroxyalprazolam: 4-OH alprazolam) were measured by HPLC/UV.

The production of 4-OH alprazolam, one main metabolite of alprazolam, was weakly inhibited by higher dose of ethanol, but not α-OH alprazolam.

These results using a human liver microsomal preparation show that the production of 4-OH alprazolam is weakly inhibited by ethanol but not α-OH alprazolam. Toxic levels may be reached by simultaneous administration of ethanol and high-dose alprazolam.

Keywords: Drug interaction; Cytochrome P450; Ethanol; Alprazolam; *In vitro*

1. Introduction

Many adverse drug–drug interactions of toxicological and clinical interest appear to be attributable to pharmacokinetic changes that can be understood in terms of alterations in hepatic drug metabolic pathways catalyzed by the cytochrome P450 (CYP) system. The two major causes of drug interactions involving the CYP system are induction and inhibition, with inhibition appearing to be more important as far as documented toxicological and clinical problems are concerned.

Alprazolam is a triazolobenzodiazepine derivative, mainly used as an short-acting antidepressant and anxiolytic agent, and its effect appears at low doses,[1] while ethanol is one of the most commonly used social drugs in many countries. Sometimes, toxic interactions occur following combined administration of these two drugs.[2] We have already described the toxicological interactions between ethanol and two benzodiazepines.[3,4] Fatal or serious overdosing involving coadministration of benzodiazepines, especially alprazolam, continues to be a major problem.[5–8] There is no published report of a pharmacokinetic interaction between ethanol and alprazolam *in vitro*.

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In this study we investigated the *in vitro* interaction between ethanol and alprazolam and its two main metabolites (α-hydroxyalprazolam: α-OH alprazolam, 4-hydroxy alprazolam: 4-OH alprazolam) at high-dose concentrations using human liver microsomes.

2. Methods

2.1. Chemicals

NADPH was purchased from Oriental Yeast Co. (Tokyo, Japan). Diazepam (internal standard, I.S.) and ethanol were purchased from Wako (Osaka, Japan). Alprazolam and its two metabolites (α-OH alprazolam and 4-OH alprazolam) were kindly provided by Roche (Basel, Switzerland). All other chemicals and reagents used were of the highest commercially available quality.

2.2. Human liver microsomes

Microsomes from three pooled human livers (Catalog No: H003, H013, H032) containing representative activities of CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 were obtained from Daiichi Pure Chemical Co. (Tokyo, Japan).

2.3. Enzyme assay

The incubation mixture contained protein (0.125 mg), 0.1 M potassium phosphate buffer (pH 7.4), 0.1 mM NADPH, alprazolam (substrate concentration: 0.5–80 nM; therapeutic level 65–195 nM, toxic level 325–1300 nM)[9] and ethanol (0–80 mM; toxic level 20–40 mM, fatal level 70–80 mM) in a total volume of 0.5 ml. Incubations were initiated following a 3-min preincubation at 37 °C by the addition of NADPH and generally carried out for 20 min in a shaking water-bath at 37 °C. The reaction was terminated by adding 100 μl acetonitrile and 3 ml *tert*-butyl methyl ether containing 0.25 μg/ml thiamylal (IS). After vortex mixing for 3 min, the tubes were centrifuged at 1200*g* for 5 min. The organic phase was transferred to a clean conical tube and evaporated in a water-bath at about 40 °C under a gentle stream of nitrogen. The residue was dissolved in 200 μl mobile phase and 50 μl injected into the HPLC system. The substrate was delivered in a 0.1% (final concentration, v/v) solution of methanol.[6]

2.4. Determination of alprazolam and its two metabolites

The HPLC equipment consisted of a pump (Model CCPS, Tosho, Tokyo, Japan) and a variable-wavelength UV detector (Model UV-8020, Tosho, Tokyo, Japan). Separation was achieved using a C_{18} reversed-phase column (150 mm × 4.6 mm I.D.), particle size 3 μm, Inertsil ^{R}ODS-3 (GL Sciences Inc., Tokyo, Japan). The mobile phase was 20 mM KH_2PO_4–methanol–acetonitrile (40:20:30, v/v/v) and the flow rate was 0.6 ml/min. The absorbance of the eluent was monitored at 254 nm. All instruments were operated at ambient laboratory temperature (ca. 23 °C). The retention time of α-OH alprazolam, 4-OH alprazolam, alprazolam and the IS extracted from a spiked sample of human liver microsomes was 9.6, 10.5, 15.0 and 24.5 min, respectively. The accuracy and precision values for the analytical procedure were within 10%. The limits of detection (LOD) and quantification (LOQ) of α-OH alprazolam and 4-OH alprazolam were 20 and 25, and 10 and 15 μM, respectively. The intra- and inter-assay coefficients of variation (CV) for the drug and its metabolites were less than 5.3%. The accuracy (%) and precision (CV %) values of recovery rate of two metabolites for this procedure were approximately 97% and 6.2%, respectively.

2.5. Statistical analysis

To determine significant differences between group mean values, data were examined using a one-way ANOVA test for repeated measures. Differences were considered significant when $p < 0.05$. Results are expressed as the mean ± SE.

3. Results and discussion

Serious toxic interactions or side-effects occur following the combination of some drugs. The purpose of this study was to investigate the metabolic interaction involving alprazolam metabolites and ethanol using human liver microsomes over the therapeutic range *in vitro*.

Alprazolam is a substrate of human CYP3A isoforms, mainly CYP3A4/5, present in both liver and gastrointestinal tract mucosa and two main metabolites are produced.[10,11] The metabolites, α-OH alprazolam and 4-OH alprazolam, exhibit approximately 66% and 19% of the potency of the parent drug, respectively.[12] Its metabolism is inhibited by fluvoxamine, citalopram and fluoxetine, *in vivo* and/or *in vitro*.[13,14]

Ethanol is metabolized by the liver, the major site of ethanol metabolism, and hepatic alcohol dehydrogenase (ADH) is the enzyme primarily responsible for this process. Long-term intake of large amounts of ethanol induces pathways of metabolism that are independent of ADH.[15–19] Other enzymes, in particular the microsomal ethanol-oxidizing (MEOS) system, involving CYP2E1, are also involved at higher doses of ethanol, and these metabolize up to 10% of the ingested ethanol.[15–19] Acute ethanol ingestion causes competitive inhibition of the enzymes involved in ethanol catabolism, including CYP1A2, CYP2E1 or CYP3A,[15–17] while chronic exposure to ethanol may lead to induction.[18,19]

In their drug–drug interaction study of acute alcohol ingestion *in vivo*, Linnoila et al.[20] investigated the effects of alprazolam, alone and in combination with ethanol (0.8 g/kg), on the pharmacodynamics and pharmacokinetics of the drugs in healthy male volunteers. Alprazolam 2 mg produced relatively long-lasting impairments in tests

Table 1
Effect of ethanol on alprazolam metabolism in human liver microsomes *in vitro*

Drug and its metabolites	Ethanol concentration			
	Control (%)	20 mM (%)	40 mM (%)	80 mM (%)
Alprazolam (0.5 μM)				
α-OH	12.4 ± 3.4(100)	12.7 ± 3.7(102)	14.6 ± 2.7(118)	18.2 ± 1.9(147)
4-OH	26.8 ± 5.3(100)	21.7 ± 4.1(81)	22.7 ± 1.0(85)	18.7 ± 2.6(70)
Alprazolam (1 μM)				
α-OH	12.5 ± 1.6(100)	14.5 ± 2.9(116)	18.2 ± 2.9(146)	19.3 ± 3.6(154)
4-OH	59.4 ± 5.2(100)	50.6 ± 7.6(85)	54.0 ± 6.4(91)	47.8 ± 5.8(80)
Alprazolam (2 μM)				
α-OH	12.9 ± 1.4(100)	20.0 ± 2.2(155)	19.6 ± 2.7(152)	21.1 ± 2.9(163)
4-OH	113.1 ± 20.2(100)	120.2 ± 14.7(106)	110.0 ± 19.0(97)	95.8 ± 13.3(84)
Alprazolam (4 μM)				
α-OH	27.0 ± 4.6(100)	26.0 ± 4.8(96)	24.9 ± 2.7(92)	25.6 ± 3.9(95)
4-OH	254.1 ± 40.4(100)	218.9 ± 26.8(86)	199.5 ± 29.3(79)	203.1 ± 42.6(80)

Values are the mean ± SE of triplicate determinations, nmol/min/mg protein, alprazolam and its two main metabolites (α-hydroxyalprazolam: α-OH alprazolam, 4-hydroxyalprazolam: 4-OH alprazolam) were measured by HPLC/UV.

of tracking, verbal and nonverbal information processing, and memory, and reduced blood pressure without a change in heart rate or plasma norepinephrine levels. However, the plasma concentration of alprazolam remained unchanged. In a fatal case, Michaud et al.[21] described a 30-year-old woman, with a history of depression, who was found dead after the ingestion of an unknown quantity of alprazolam, tramadol and alcohol. The blood concentrations of alprazolam, alcohol and tramadol were 0.21 μg/ml, 1.29 g/kg and 38.3 μg/ml, respectively. In this case, it was not possible to ascribe the death only to tramadol because of the high levels of ethanol and alprazolam.

There are few published interaction studies involving ethanol and alprazolam *in vitro*. In this *in vitro* study, the production of 4-OH alprazolam, one main metabolite of alprazolam, was weakly inhibited by higher dose of ethanol (15–30%), but not α-OH alprazolam (10–20%) (Table 1). The reasons for this discrepancy may be a difference in affinity for the drug metabolizing enzyme involved in the production of α-OH alprazolam and 4-OH alprazolam, although this was not investigated in the present study. These results may be due to an increase in the blood alprazolam level after coadministration of higher doses of both ethanol and alprazolam.

We have already described the *in vitro* interaction between alcohol, and triazolam[3] and flunitrazepam[4] at therapeutic concentrations using human liver microsomes. However, in the present study we found that there was weak interaction between ethanol and alprazolam. Although the reasons for this is not apparent, the extent of this inhibition will be a function of the contribution of an enzyme to the metabolism of a compound and the affinity for the inhibitor.

In conclusion, these results using a human liver microsomal preparation show that the production of 4-OH alprazolam was weakly inhibited by higher doses of ethanol but not α-OH alprazolam. Toxic levels may be reached by simultaneous administration of ethanol and high-dose alprazolam. In future, different human microsomes will need to be used to establish the existence of CYP genetic polymorphisms and *in vivo* clinical studies will need to be carried out mainly involving toxicological alcohol–drug interactions.

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